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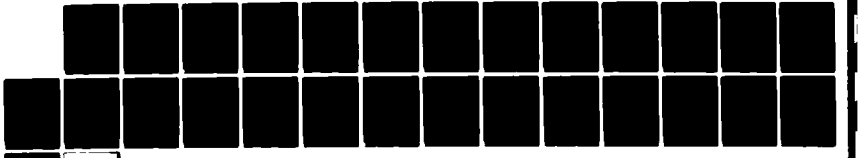

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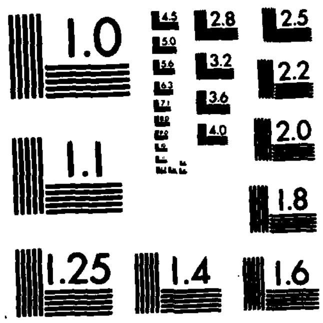

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RAPID DIAGNOSIS OF ARBOVIRUS AND ARENAVIRUS INFECTIONS BY IMMUNOFLUORESCENCE

THIRD ANNUAL REPORT

Jordi Casals-Ariet, M.D.

January 1980

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Yale University School of Medicine
New Haven, Connecticut 06510

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20. Abstract (continued)

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It was established that bloods and sera from adult mice peripherally inoculated with Banzi virus contained high concentrations of virus. Attempts were made to identify the virus rapidly with the IF technique. Blood smears failed to show specific fluorescence; dilutions of blood inoculated to cell monolayers permitted specific detection of the virus in two days.

Inoculation of cell monolayers with dilutions of a Junin virus stock, followed by processing and staining for the IF test permitted specific detection of moderately large amounts of virus 8 and 12 hours after inoculation.

The successful use of the IF test with spot-slides for sero-epidemiological surveys with Lassa fever virus was continued. Furthermore, the technique was also used with success in the identification of virus strains, by quickly giving the antigenic group affiliation, through the use of monovalent slides in combination with polyvalent reference immune reagents, or by testing a monovalent serum against polyvalent slides.

SUMMARY

Slides with 12 small circular areas 5 mm in diameter, containing cultured cells infected with a virus, spot-slides--for use as antigen in the indirect immunofluorescence (IF) test have been prepared with the following viruses: Mayaro, dengue type 3, dengue type 4, West Nile, Oropouche and Sicilian phlebotomus fever. Similar slides containing a mixture of cells infected with different flaviviruses, dengue types 1 and 2, Japanese encephalitis (JE), Rocio, Langat and yellow fever were also prepared; hyperimmune antisera for 20 distinct group B viruses were tested with the slides and all but 4 gave a positive reaction at dilution 1:8 or higher.

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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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BODY OF REPORT

I. Statement of problem

The problem under consideration is early diagnosis of arbovirus and arenavirus diseases of man, primarily serological diagnosis but, in so far as resources permit, also virological diagnosis. At the patient's level early diagnosis is desirable in as much as administration of immune plasma for treatment of arenavirus and Marburg-type diseases is determined by the diagnosis; administration of plasma very early in the course of the disease may be more beneficial than if given later and possibly less conducive to induce immunopathological complications. Chemotherapeutic substances, when available, may also be more effective when given early. At the group or community level, a rapid specific diagnosis is of the utmost importance since it determines whether or not certain measures such as vector or reservoir control, isolation or quarantine and vaccination if available, should be applied. As these measures are expensive and to some extent disruptive for the community's life, they should be applied only if an early specific diagnosis indicates that they are needed.

There is little doubt that the fastest way to accomplish a diagnosis is by visualization and specific identification of the virus or its antigens directly in clinical specimens. If this approach fails and an amplifying host system, i.e. animal or cell culture passages, is used, the time required for diagnosis may be shortened by attempting to detect the virus or antigen in the amplifying system before disease or lesions are apparent.

If the virus has not been detected, serological methods are used to determine the presence of circulating antibodies in the patient. The question addressed in this project is to explore the possibilities of a relatively new procedure, the immunofluorescence technique (IF), and compare its results with those of longer-established tests, complement-fixation (CF), hemagglutination inhibition (HI) and neutralization. Development and use in the IF test of antigen-containing slides in the form of fixed cells infected with arboviruses and arenaviruses, and the application of these slides to early diagnosis and to seroepidemiological surveys, is the main objective of this work.

II. Background

Specific diagnosis of a current arbovirus or arenavirus disease is made by isolation and identification of the virus and by detection of antibody development between acute and convalescent serum samples. Conventional procedures that involve propagation of the virus in a susceptible host system and waiting for a second serum sample for antibody tests are not conducive to rapid diagnosis.

The fastest way to secure a diagnosis is through detection of the virus directly in clinical specimens without recourse to an amplifying host. Several techniques are used in this connection: electron microscopy, immunoelectron microscopy, IF, radioimmuno-assay (RIA) and enzyme linked immuno-assays such as ELISA and immuno-peroxidase. IF has been applied to the direct detection of Lassa fever virus in patients' conjunctival scrapings with partial success: the antigen is detected in about half the number of proven cases (J.B. McCormick and K.M. Johnson, personal communication, 1979). At an earlier time, the IF test was reported as successfully detecting Colorado tick fever virus in the blood of patients (Emmons and Lennette, 1966). Electron microscopy permits the visualization of certain virions--Ebola and Marburg--directly in the blood of patients (Bowen et al., 1978; Peters and Müller, 1968); and in fatal cases the diagnosis can be made by electron microscopic examination of liver specimens. It must be borne in mind, however, that electron microscopy indicates only the family, not the serotypes involved.

Visualization of arbovirus and arenavirus virions in clinical specimens is accomplished rarely; it is generally necessary to use an amplifying host, animal or cell culture. In such case the procedure can be speeded up by identifying the virus in the inoculated host, usually by IF. This procedure has been applied to the diagnosis of dengue by inoculation of mosquitoes (Kuberski and Rosen, 1977) or tissue cultures (Digoutte et al., 1979; Tesh, 1979); and of St. Louis encephalitis virus in cell cultures (Mattos et al., 1979).

Serological tests are employed for diagnosis of arbovirus and arenavirus infections when no virus has been isolated and to complete the diagnosis even when virus is isolated. Seroconversion, as it relies on a late serum sample, is a much delayed diagnostic procedure. A valuable lead towards a specific diagnosis with a single serum sample is given when this sample has a high antibody titer. A presumptive specific diagnosis with an early serum sample is arrived at by fractionation of the immunoglobulins and detection of antibodies in the IgM fraction; furthermore IgM antibodies, or early antibodies, are more specific than the late IgG class.

In chosing an assay for rapid serological diagnosis, two elements must be considered: the time required to complete the test and the time in the course of the disease when antibodies are first detectable by the test chosen. With arboviruses, particularly alphaviruses (group A) and flaviviruses (group B) with which considerable information has accumulated, the consensus is that antibodies are detected by neutralization test, followed by HI and later by CF (Casals and Palacios, 1941; Lennette et al., 1953; Theiler and Casals, 1958; Buescher et al., 1959; Stallones et al., 1964; Galinovic-Weisglass and Vesenjak-Hirjan, 1976). With the

arenaviruses, no HI test is available and the CF antibodies develop lat; hardly any patients have been positive before 10 days from onset in LCM (Deibel et al., 1975), before 14 in Lassa fever (Monath and Casals, 1975) and before 20 days in Bolivian hemorrhagic fever (BHF) patients (Johnson, 1970). The neutralization test has not been much used as an aid to a current diagnosis with the arenaviruses; in addition to the fact that these antibodies are also relatively late in appearing, the test itself is not a simple procedure with LCM (Hotchin and Kinch, 1975) and its interpretation presents difficulties with Lassa virus (Monath, 1975).

Since about 1975 the IF test has become the choice method for serological diagnosis of arenavirus diseases and diseases caused by pathogens of the Marburg type; this technique has also been applied to some arbovirus infections. The use of IF has been reported with: Colorado tick fever (Emmons et al., 1969); BHF (Peters et al., 1973); Argentinian hemorrhagic fever (AHF) (Grela et al., 1975); Lassa fever (Wulff and Lange, 1975); Congo-Crimean hemorrhagic fever (CCHF) (Zgurskaya et al., 1975; Burney, 1979); and Marburg and Ebola fevers (Webb et al., 1978; Bowen et al., 1978).

While not the subject of investigation in this project, other serological techniques may deserve considerationfor early diagnosis of arbovirus and arenavirus diseases. The indirect hemagglutination test and its inhibition has been described for Tacaribe and LCM viruses (Gajdamovich et al., 1975). The agar gel diffusion and precipitation test has been extensively used by Soviet investigators (Chumakov and Smirnova, 1972; Karinskaya et al., 1974) for CCHF. The solid phase RIA using protein A-bearing Staphylococcus aureus as the solid phase immunoadsorbent has been successfully applied to arboviruses (Jahrling et al., 1978). The enzyme-linked immunosorbent assay (ELISA) first described by Engvall and Perlmann (1972) has been recently reported with arboviruses (Frazier and Shope, 1979).

III. Approach

The working hypothesis was made at the outset of this project that a rapid specific diagnosis of arbovirus and arenavirus infections could be made in a simple way by means of the IF technique. This hypothesis was to be tested by applying the technique to the detection of antibodies, i.e., serological diagnosis; and to the detection of antigen, i.e., virological diagnosis.

The latter requires availability of clinical specimens obtained early in the course of a disease. Considering that easy access to such materials was not a realistic proposition given our circumstances, the test of the hypothesis was to be accomplished by producing the disease in small laboratory animals and securing the equivalent of clinical specimens--in fact, bloods or sera--in the period immediately following infection.

Ways for rapid serological diagnosis can be readily evaluated through the use of sera from animals inoculated once or repeatedly and bled at intervals thereafter. The working hypothesis can be, and has been, submitted to a real test by using field collected human sera from both current disease cases and in seroepidemiological surveys; the test is being done on a continuing basis through the use of antigens consisting of cells from infected tissue cultures deposited on microscope slides, fixed with acetone (spot-slides) and stored at low temperature.

IV. Materials and Methods

Viruses. The viruses and strains used were: Mayaro, Tr 4675; Banzi, SA H336; dengue type 1, Hawaii; dengue type 2, NGB; dengue type 3, H 87; dengue type 4, H 241; JE, Nakayama; Langat, TP 21; Rocio, San Paulo; West Nile, Egypt 101; yellow fever, Asibi; Oropouche, Tr 9760; Sicilian phlebotomus fever, Sabin; and Junin, XJ vaccine strain. The viruses are maintained as 10% infected newborn mouse brain tissue suspensions held lyophilized or wet frozen at -60C.

Cell cultures. The following cell cultures routinely maintained in this laboratory have been used: VERO, BHK-21, LLC-MK2, and CER (Smith et al., 1977). The cells are maintained usually in 150 cm^2 plastic bottles; transfers are made every 8-10 days for VERO and LLC-MK2, every 4-6 days for the others. At long intervals fresh cultures are started from cells held in liquid nitrogen.

Animals. The following have been used for preparation of antisera or to develop models of infection: mouse (Mus musculus), Swiss outbred; hamster (Mesocricetus auratus); guinea pig (Cavia porcellus); and rabbit (Oryctolagus cuniculus). These animals were purchased from commercial dealers; mice were used as adults or as newborn, as required.

All animals were bled or intracerebrally (IC) inoculated under deep ether anesthesia. Whole bloods or sera were kept individually except with mice; they were bled in groups of 2 to 4 and the bloods pooled in order to insure sufficient working volumes. Bloods and sera for virus detection were stored at -60C; sera for antibody tests at -20C.

Preparation of spot-slides. The method of preparation was described in detail in the Second Annual Report, January 1979; in essence the method is as follows.

Cell cultures were infected with a given virus at a MOI from 100 to 1. As soon as CPE is 1 or 2 plus, or after a predetermined number of days with viruses that replicate without CPE, the cells were harvested, dispersed with trypsin and versene, washed in

phosphate buffered saline (PBS) and adjusted to a count of 3×10^6 cells/ml; a similar suspension was prepared with uninfected cells. The two suspensions were mixed in a proportion of 3 parts infected cells to 1 part uninfected; the cell mixture in volumes of 0.01 ml (or 3×10^4 cells) was dropped on the surface of teflon-coated slides (Cel Line Associates, Minotola, New Jersey) which have 12 circular areas 5 mm in diameter. The drops were allowed to dry at 37C, the slides were immersed in acetone for 10 minutes and stored at -60C. Slides have been used from 1 1/2 to 2 years after preparation with excellent results.

Preparation of chamber slides. Tissue culture slides 75 x 25 mm with 4 chambers were used (Lab-Tek Products, Miles Laboratories, Neperville, Illinois); the capacity of the chambers was 1 ml. Monolayers under fluid medium were prepared by seeding each chamber with 2 to 2.5×10^5 cells in a volume of 0.8-0.9 ml of growth medium; the slides were held in a CO_2 incubator. One to 3 days later the monolayers were infected with the virus, or with a virus-serum mixture for a neutralization test, in a 0.1 ml volume; maintenance fluid was added after a period of adsorption of 1 hour and the chambers held at 37C in a CO_2 incubator. At the desired time after incubation the chambers were drained and removed, and the slides fixed in acetone for IF staining.

Preparation of micro-cell cultures. These cultures were prepared by means of a new commercially available product (Bellco micro-slide culture chamber). The assembly consists of a silicone-rubber block or matrix 55 x 25 x 12 mm having 10 cylindrical perforations, about 5-6 mm in diameter. A microscope slide is placed under the openings on one side of the block and a metal plate with matching holes on the other side; the whole is held together with 2 lateral clips with the result that 10 wells, each with a capacity of 0.4 ml are formed, each well becoming a culture chamber.

In our experience the chambers have been seeded with from 5000 to 20000 cells in 0.3 ml of medium; 24 or 48 hours later the monolayer is ready to be infected. We have found it advisable to use teflon-coated slides, instead of the ordinary slides supplied by the manufacturer, to prevent reagents from running all over the slide when the chamber is disassembled for IF staining. The processing and fixing of these micro-cell cultures is the same as used with chamber-slides. Although only exploratory tests have thus far been done with these micro-cell cultures, their usefulness appears promising.

Immunofluorescence test. Fluorescent conjugates were purchased from commercial dealers; preparations used routinely in this laboratory were conjugated anti-globulins for man, mouse, hamster, guinea pig and rabbit. The indirect IF technique used was a standard laboratory procedure (Gardner and McQuillin, 1975);

Evans' blue at a final dilution of 1:10000 was used as a counter stain. An Olympus "Vanox" incident light microscope set for blue fluorescence was used; at first, a xenon light source was employed, more recently a mercury one. When viewed with X40 or X100 objectives, glycerol placed directly over the slide without a cover-slip was used.

Other serological tests. Other methods used, CF, HI and neutralization tests in mice or in cell cultures, have been described in detail (Casals, 1967). The CF and HI tests were semi-micromethods with a total volume of reagents of 0.15 ml and 0.1 ml, respectively. The antigens were prepared from infected newborn mouse brain tissue extracted with acetone and sucrose; or from infected tissue cultures.

V. Results

Polyvalent spot-slides. Polyvalent spot-slides were prepared for the flavivirus genus (antigenic group B). Ideally these slides should react positively with all sera containing antibodies against any virus of the group. Considering that there are currently over 50 flaviviruses and that, for technical reasons, slides containing more than 4×10^4 cells in a spot are unsatisfactory due to over-crowding, it follows that only a limited number of viruses can be directly represented in the slide. Otherwise the number of cells infected with each virus will be so small that even when strongly positive may be time-consuming to find. The practical use of the polyvalent group B slides is based on cross-reactivity among the viruses in the group.

With these considerations in mind, it was decided to prepare slides with 6 viruses representing different complexes in the group; allowing 4×10^4 cells in a spot, each virus would be represented by about 6500 infected cells, or somewhat fewer if not all the cells in the culture were infected. Much effort went into finding the optimal cells for each virus and in co-ordinating the days on which the individual viruses were to be inoculated, in view of the differences in length of incubation needed in order to attain a CPE between 1 and 2 plus on the same day. The outcome of the preliminary explorations was to use the following viruses, cells and time of infection prior to harvesting: Japanese encephalitis, VERO, 3 days; Rocio, VERO, 5 days; yellow fever, VERO, 4 days; a nearly equivalent mixture of dengue types 1 and 2, LLC-MK2, 7 days; and Langat, BHK-21, 5 days.

The infected cells were processed separately according to virus, the cell suspensions adjusted to a count of 4×10^6 cells/ml and mixed in equal parts thus making a suspension containing 0.8×10^6 cells/ml infected with each virus; spot-slides were prepared with the cell mixture in the routine manner. The capacity of the slides to detect antibodies against flaviviruses was tested with hyperimmune mouse sera or ascitic fluids; the result of a test is shown in Table 1.

The quality and intensity of the IF stain was satisfactory with most of the antisera; however, four reagents gave negative reactions at dilution 1:8. Possibly two of the antisera, Banzi and Tembusu, may have had low homologous titers. While these polyvalent slides seem to be reasonably satisfactory, it may be advisable in the future to prepare two sub-sets of group B polyvalent slides, each with 5 different viruses, in order to increase the probability of complete coverage.

Rapid diagnosis of arbovirus infection: Banzi virus viremia in mice. The initial phases of this study were reported in the Second Annual Report, January, 1979. As indicated then, the basis for this investigation was the observed fact that inoculation of small amounts of Banzi virus intraperitoneally to adult mice results in an infection characterized by a 6-day incubation period, followed by disease on the 7th or 8th day and death shortly after. Assuming that viremia were present during the incubation period and during the disease, this simple and reproducible experiment would be usable as a model to investigate how to diagnose rapidly the specific nature of an arbovirus infection. Groups of 3 mice were bled daily, beginning with day 1 after inoculation, thick blood smears made, a small amount of whole blood hemolyzed by mixing with 2 volumes of water and from the remaining blood the serum was separated; all these materials were stored at -60C.

Results of viremia titrations in whole blood were reported in the Second Annual Report. At that time, in view of the high susceptibility of adult mice to this virus inoculated intraperitoneally, we reasoned that a sensitive test for viremia might be the intraperitoneal inoculation of 1 ml of blood in dilutions, a much larger volume than can be injected intracerebrally. The maximum titer observed by this method was $10^{3.5}$ LD_{50}/ml, on the 2nd post-infection day.

This titer seemed low, certainly much too low to expect success in detecting the virus directly in the blood by any available method. On the assumption that the method used, IP titration in adult mice, was not as sensitive as it was at first thought to be, the titrations of viremia were repeated by IC inoculation of 0.02 ml to 2- to 4-day-old mice; since not enough samples of blood were left, the titrations were done on the sera. The viremia titers noted, considerably higher than those first observed, are given in Table 2.

Traces of virus were detected 1 day after infection, with 2 of 8 inoculated mice dying at dilution 10^{-1}, lowest used; beginning with day 2 and until day 5, the titers of virus expressed for 1 ml, rather than for the inoculated 0.02 ml amount, were between $10^{4.1}$ on day 2 and $10^{3.8}$ on day 5, with a maximum of $10^{6.5}$ on day 3. During this time the mice had appeared normal. On dav 6, only traces of virus were recovered, with 1 of 8 mice succumbing;

no virus was detected on day 7 when the mice were sick of on day 8 when they were moribund. These titers are not strictly comparable to those reported in the Second Annual Report in that last year's were obtained with whole blood, while this year's are derived from serum; whether the source--blood or serum--is responsible for the difference or the different technique used--IP in adult mice versus IC in newborn mice--remains to be ascertained.

In view of the high titers observed in the serum of infected mice, it would appear that Banzi virus peripherally inoculated to adult mice constitutes a useful model for investigation of methods for early and rapid detection of circulating antigen in arbovirus diseases.

Early detection of Banzi virus in viremic mice by IF.

i. Thick smears of blood taken from viremic mice, as described in the preceding section, fixed in acetone or methyl alcohol were processed for indirect IF using a guinea pig anti-Banzi immune serum and a normal guinea pig serum as control. There were difficulties in maintaining the blood attached to the slides during the repeated washings; as far as the experiments went, no difference was noted between smears treated with immune and normal serum.

ii. An attempt was made to determine the correlation between the development of viremia in the infected mice and the time that it would take to recognize this viremia by means of the IF test in an amplifying system, in this instance cell cultures inoculated with viremic bloods. Dilutions 1:3, 1:30 and 1:300 of hemolyzed whole blood from days 1, 2, 3 and 4 after infection were inoculated to a sufficient number of BHK-21 monolayers in Lab-Tek chamber-slides. Slides inoculated with each daily blood were processed and stained for IF examination on each of four consecutive days--1 to 4 after inoculation--using a Banzi mouse hyperimmune serum and the indirect IF test; the results are shown in Table 3.

As Table 3 shows, first day blood gave negative IF staining throughout; this blood was also negative by mouse inoculation (see Table 2). Second day blood at dilution 1:3 gave positive IF in monolayers held 2 or more days. Third and 4th day bloods first gave a positive IF reaction 24 hours after inoculation of the monolayers, but only at dilution 1:3; when these monolayers were held 2 or more days after inoculation all the blood dilution gave positive IF. These results show that a specific diagnosis of Banzi viremia can be made with blood taken on the 2nd day or later, with monolayers incubated no more than one or 2 days.

Early detection of antigen by IF. In addition to Banzi virus in the blood of viremic mice, other viruses were tested with a view to correlate in an amplifying cell culture system the result of the

IF test, the time required for the test's completion and the amount of virus in the material being tested. Initial results were given in the Second Annual Report, January 1979, pages 13 and 14.

i. Japanese encephalitis virus. Dilutions of a mouse brain virus stock of JE, Nakayama strain, were inoculated to VERO cell monolayers; on successive days after inoculation monolayers were processed for IF tests, after recording the presence and degree of CPE. The result of an experiment is shown in Table 4.

As shown in the table, when large amounts of virus were inoculated, from 10^5 to 10^7 mouse $ICLD_{50}$, the IF reaction was strongly positive in monolayers after 24 hours. As little as 10 LD_{50} of virus was specifically detected 3 days after inoculation of the monolayer, whereas 5 to 7 days were necessary for CPE to appear; considering the known incubation period of this virus at that dilution in newborn mice, probably from 4 to 6 days would be required for the animals to come down with visible disease. In either case, positive CPE at 5 days or positive mouse inoculation at 4 to 6, the specificity of the agent would still remain undetermined, whereas IF as described gave the answer in 3 days.

ii. Junin virus. As described in the Second Annual Report, January 1979, as little as 1.2×10^2 newborn mouse $ICLD_{50}$ of the virus could be specifically detected in 24 hours, in an experiment in which dilutions of a stock suspension were inoculated to VERO cell monolayers in Lab-Tek chamber-slides. Additional investigations have been done with the same system--Junin virus in dilutions propagated in VERO cells--narrowing the observations to within 12 hours after inoculation. Monolayers were inoculated, as before, with dilutions of Junin virus, held at 37C for 1 hour to allow for virus attachment, then processed at 0, 1, 2, 4, 8 and 12 hours; a set of monolayers was processed 7 days after inoculation, as positive control. The result of the experiment is given in Table 5.

As seen in Table 5, eight and 12 hours after inoculation the slides unmistakably showed the presence of Junin virus by IF in amounts of 1.2×10^3 and 1.2×10^2 $ICLD_{50}$, respectively. In addition, a definite stippling in the cytoplasm of from a few to many cells was observed at 1, 2 and 4 hours following inoculation of the monolayers with the larger doses of virus; while it would be unreasonable to base a positive diagnosis of Junin virus infection on this finding alone, its potential implications are being pursued.

Applications of the IF test to arbovirus and arenavirus diagnostic problems. The use of the IF technique in current investigations in this laboratory has continued to prove its practical value.

i. Lassa fever virus. A survey to determine the presence of IF antibodies against this virus in hospital personnel in Africa, is being conducted on a continuing basis in association with Dr. J.D. Frame, College of Physicians and Surgeons, Columbia University, New York, N.Y. Between January 1 and December 15, 1979, 77 sera originating in Liberia and Uganda were tested; 6 were positive and 1 questionable, all from Liberia.

ii. Virus identification. Two viruses, F-41451 and Aus-CF 122, partially identified at the originating laboratories, were submitted to YARU for final identification. As a preliminary step, confirmation of their antigenic group affiliation was sought by means of the IF technique, in part in order to determine the applicability of the test to this kind of problem.

F-41451 was inoculated to several cell lines, including BHK-21, VERO and CER; at the proper time, spot-slides were prepared and tested by IF against dilution 1:4 of the following polyvalent mouse ascitic fluids: group A, group B, groups California, phlebotomus fever, Sakhalin, VSV and NIH polyvalent #1, 2, 4, 5, 6 and 9. Only polyvalent group A fluid reacted positively, in all the cell lines, which clearly gave the antigenic group affiliation of the virus.

An immune mouse serum was available for virus CS 122; the serum in dilutions beginning at 1:4 was tested by IF against polyvalent slides for groups A and B. The reaction was positive, although weak, for group B at serum dilutions 1:4 to 1:16; entirely negative with the polyvalent group A slide. The grouping of this strain, given the serum and the slides, was accomplished in 3 hours.

iii. Antigenic relationships involving CCHF, Nairobi sheep disease and other tick-associated viruses. Recent studies in this laboratory have revealed the existence of a conglomerate of distantly related antigenic groups of arboviruses; the IF test is proving to be an important method for the elucidation of these relationships. The type of relationship thus far elicited among the group is similar to the one noted a few years ago, also in this laboratory, which led to the establishment of the Bunyamwera supergroup and eventually, when physico-chemical methods were applied, to the definition of the genus Bunyavirus. The antigenic groups which on-going studies have shown to be distantly related are: Congo-Crimean hemorrhagic fever, Nairobi sheep disease, Dera Ghazi Khan, Bandia and Hughes. The position of the viruses in these groups within the system of classification adopted by the International Committee on Taxonomy of Viruses is not clear, mainly for lack of data. Whether this new supergroup, Nairovirus supergroup, will become another genus in the family Bunyaviridae; or a new family distinct from the Bunyaviridae awaits molecular virology studies.

Supply of spot-slides for IF tests to USAMRIID. As part of the contract agreement, spot-slides were prepared, tested for specificity and adequacy and shipped to USAMRIID; two ampules of the corresponding virus stocks and 6 x 0.5 ml of hyperimmune mouse serum were included in the shipment. The viruses and cells used and the number of slides supplied were:

Dengue 3, H 87, LLC-MK2; 200
Dengue 4, H 241, LLC-MK2; 200
Mayaro, Tr 4675, VERO; 200
Polyvalent group B, several; 260
Oropouche, Tr 9760, VERO; 200
West Nile, Eg 101, VERO; 200
Sicilian phlebotomus fever, CER; 200

VI. Discussion

The need for specific rapid diagnosis of arbovirus and arenavirus diseases and, even more so of diseases caused by the Marburg-Ebola type viruses is becoming increasingly urgent as the occurrence of outbreaks of these diseases seems to be more frequent. The reason for the desirability of an early diagnosis is, in view of the propensity of some of these diseases for person-to-person spread, the need for implementing the adequate measures for the treatment and management of the patient, as well as for protection of hospital staffs, relatives and the community at large; in the military establishment the well-being and efficiency of a body of troops may depend on an early diagnosis.

The optimal answer is, undoubtedly, direct recognition of the pathogen in clinical materials, blood, secretions or excretions. This has been accomplished with the Marburg and Ebola viruses through electronmicroscopy. Whether a similar type of diagnosis is possible with arboviruses and arenaviruses using IF test, only time will tell. It is conceivable that with a disease like Rift Valley fever in man, when titers of viremia of the order of 10^9 or 10^{10} LD_{50} doses per ml have been reported, sedimentation of the virus from a reasonable volume of serum on to a surface to which the virus attaches and IF staining, may allow visualization of the antigen.

The use of an amplifying host, usually a cell culture, in conjunction with IF of the inoculated cultures has shown its value in several instances, principally with Lassa fever. The findings reported here, as well as those of other workers, have shown that precious time can be saved by applying the IF technique to micro cell cultures with daily observations; undoubtedly the time required for a specific diagnosis can be shortened by several days.

If detection of the antigen fails, the serological diagnosis of an arbovirus or arenavirus disease may be speeded up by the use of IF. In certain diseases, particularly those caused by arenaviruses, a serological diagnosis is generally much delayed due to the fact that currently available tests detect antibodies very late--CF test--or the test is unreliable or difficult to complete--neutralization test; the IF test with spot-slides as antigen may be very useful in this case.

The application of IF with spot-slides to seroepidemiological surveys has definite possibilities with viruses for which: a hemagglutination-inhibition test is not available; the CF test is not sensitive enough; and the neutralization test--requiring the use of live virus--is too dangerous to carry out in a containment facility less than class 4. When slides rendered avirulent by means that do not alter their antigenicity become available, the IF test should be tried. These surveys would be of particular importance with viruses of the Ebola-Marburg type, the distribution of which is practically unknown and for arenaviruses like Lassa and Junin about which much more should be known.

VII. Conclusions

1. It is feasible to prepare polyvalent spot-slides containing 6 flaviviruses, for use as antigen in the indirect immunofluorescence test. These slides react positively with most hyperimmune sera tested belonging in group B, many of which are not represented in the slides. In view, however, of the large number of flaviviruses--over 50--it is recommended that two subsets of group B slides be prepared in the future, thus considerably increasing the coverage.

2. Direct detection of an arbovirus or arenavirus in the blood of a viremic host may not be easily accomplished by the IF technique, for lack of antigenic mass; however, the time needed for its recognition by means of an amplifying host--cell monolayer--is considerably shortened through the use of micro cell cultures and daily, or during the first day, hourly IF staining.

3. Determination of antigenic group affiliation in the course of identification of a virus strain can be speedily accomplished by means of the IF reaction and the use of spot-slides prepared with the unknown strain or, conversely, by testing an antiserum for the unknown against polyvalent slides.

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Table 1

Immunofluorescence test with group B polyvalent slides

Antiserum	Titer	Antiserum	Titer
CETBE	1:16+	Phnom Pehn Bat	0
Banzi	0	Powassan	1:16+
Dengue 1	1:16+	RSSE	1:16+
Dengue 3	1:16+	Spondweni	0
Dengue 4	1:16+	SLE	1:16+
Edge Hill	1:16+	Tembusu	0
Ilheus	1:16+	Wesselsbron	0
KFD	1:16+	West Nile	1:16+
MVE	1:16+	Yellow fever	1:16+
Omsk HF	1:16+	Zika	1:16+

0 = reaction was negative at serum dilution 1:8, lowest used.

Table 2

Banzi virus: viremia titrations in adult mice IP inoculated with 150 $ICLD_{50}$ of virus, bled on successive days after inoculation

Mice bled, days after infection	Result of inoculation* Dilution of serum 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer in $ICLD_{50}$/ml
1	2**	0	0	0			$10^{2.4}$ or less
2	8	5	2	0	0	0	$10^{4.1}$
3	10	10	9	4	0	0	$10^{6.5}$
4	8	8	8	5	0		$10^{5.8}$
5	8	3	2	1	0		$10^{3.8}$
6	1	0	0	0	0		$10^{2.3}$ or less
7	0	0	0	0	0		$10^{2.2}$ or less
8	0	0					$10^{2.2}$ or less

*Titrations done in 2- to 4-day-old mice by IC inoculation of 0.02 ml of serum dilutions.

**Mice dead of 8 inoculated, except on day 3 when 10 mice were inoculated.

Table 3

Banzi virus: viremia in mice IP inoculated. Detection of antigen by IF in BHK-21 monolayers following blood inoculation

Monolayer processed on day	Day when blood was taken, and dilution 1:											
	1			2			3			4		
	3	30	300	3	30	300	3	30	300	3	30	300
1	0	0	0	0	0	0	+	0	0	+	0	0
2	0	0	0	+	0	0	+	+	+	+	+	+
3	0	0	0	+	0	0	+	+	+	+	+	+
4	0	0	0	+	0	0	+	+	+	+	+	+

Table 4

Relationship between amount of Japanese encephalitis virus and time of appearance of a positive IF reaction in VERO cell monolayers

Inoculum, 0.1 ml		Days after inoculation									
Dilution of stock	Mouse $ICLD_{50}$ estimated	1		2		3		4		7	
		IF	CPE	IF	CPE	IF	CPE	IF	CPE	IF	CPE
10^{-3}	10^{7}	4	0	4	0	4	3	4	3	4	3
10^{-5}	10^{5}	3	0	4	0	4	3	4	3	4	3
10^{-7}	10^{3}	0	0	3	0	4	$\pm$	4	3	4	3
10^{-9}	10^{1}	0	0	0	0	3	0	3	0	4	2

IF and CPE: 4, equals maximum value; 0, is negative.

Table 5

Relationship between amount of Junin virus inoculated and time of appearance of a positive IF reaction in VERO cell monolayers

Inoculum, 0.1 ml Dilution of stock	Newborn mouse $ICLD_{50}$	Hours after inoculation*						
		0	1	2	4	8	12	7 days
10^{-2}	1.2×10^{5}	0	St**	St	St	+	+	+
10^{-3}	1.2×10^{4}	0	0	0	St	+	+	+
10^{-4}	1.2×10^{3}	0	0	0	0	±	+	+
10^{-5}	1.2×10^{2}	0	0	0	0	0	+	+
10^{-6}	1.2×10^{1}	0	0	0	0	0	0	+
10^{-7}	1.2×10^{0}	0	0	0	0	0	0	+

* Time in hours after 1 hour at 37C for attachment.

**St, stippling in the cytoplasm of a number of cells.

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